Tanshinone IIA inhibits human breast cancer cells through increased Bax to Bcl-xL ratios

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Received April 9, 2008; Accepted June 4, 2008

DOI: 10.3892/ijmm_00000030

Abstract. Tanshinone IIA ($C_{19}H_{18}O_3$) was extracted from danshen *(Salviae miltiorrhizae radix)*. It has cytotoxic properties and induces apoptosis in many human cancer cells. The molecular mechanisms are poorly understood, therefore, in the present study, we aimed to elucidate its anticancer activity on human breast cancer MDA-MB-231 cells. The cytotoxic effects of tanshinone IIA on MDA-MB-231 cells were measured by MTT assay. The percentages of cells in different cell cycle phases were determined by flow cytometry. The protein expression of Bax and Bcl-2 was examined using Western blotting. The results showed that tanshinone IIA inhibits the proliferation of MDA-MB-231 cells in a dose- and time-dependent manner. Tanshinone IIA induces apoptosis in a dose-dependent manner and the percentage of cells in sub-G1 phase. It increases the protein expression of Bax but decreases the Bcl-2 expression in MDA-MB-231 cells. Our findings suggest that tanshinone IIA can inhibit the proliferation of MDA-MB-231 cells by active apoptosis. One of the mechanisms may be through up-regulating the expression of Bax but down-regulating Bcl-2 expression and then inducing apoptosis. In conclusion, tanshinone IIA has therapeutic potential in breast cancer patients.

Introduction

Danshen first appeared in the Shennong Bencao Jing (ca. 100 A.D.) and was widely used in ancient Chinese medicine (1). Tanshinone IIA is derived from danshen *(Salviae miltiorrhizae radix)* and was first described by researchers in 1968. It is known to have anticancer, (2) antioxidant, (3) and anti-inflammatory properties (4). It is well documented that tanshinone IIA can induce apoptosis in some human cancer cells, such as leukemia, (5) human hepatocellular carcinoma, (6) and nasopharyngeal carcinoma cells (7). The anticancer activity of tanshinone IIA on the human breast cancer cell *in vitro* and *in vivo* is also documented (8). However, the molecular mechanisms are not yet elucidated. In the present study, we evaluated the effects and molecular mechanisms of Tanshinone IIA on MDA-MB-231 cells.

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Key words: tanshinone IIA, Bax, Bcl-2, breast cancer

Materials and methods

Chemicals and reagents. Tanshinone IIA was purchased from Herbasin Co. (Shenyang, China). Aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide (PI), sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and MTT[3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-diphenytetrazoliumromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). L-15 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human breast cancer cell line (MDA-MB-231). The human breast cancer cell line MDA-MB-231 was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C in a humidified atmosphere (CO_2 was not present), in an L-15 medium (Sigma), containing 10% heat-inactivated FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin). The data presented in this report are from a minimum of three independent experiments.

Effects of tanshinone IIA on the viability of MDA-MB-231 cells. The MDA-MB-231 cells were plated in a density of $1x10^5$ cells/well and grown for 24 h. Various concentrations of tanshinone IIA were added and cells were grown for 24, 48 and 72 h. Only DMSO 0.2% (solvent) was added to the control regimen to determine cell viability. After 24, 48 and 72 h of culture, the viability was evaluated by MTT assay (9) in triplicate. Briefly, MDA-MB-231 cells were seeded in a 6-well plate at a density of $1x10^5$ cells/well and allowed to adhere overnight. After removing the medium, 2000 μl of

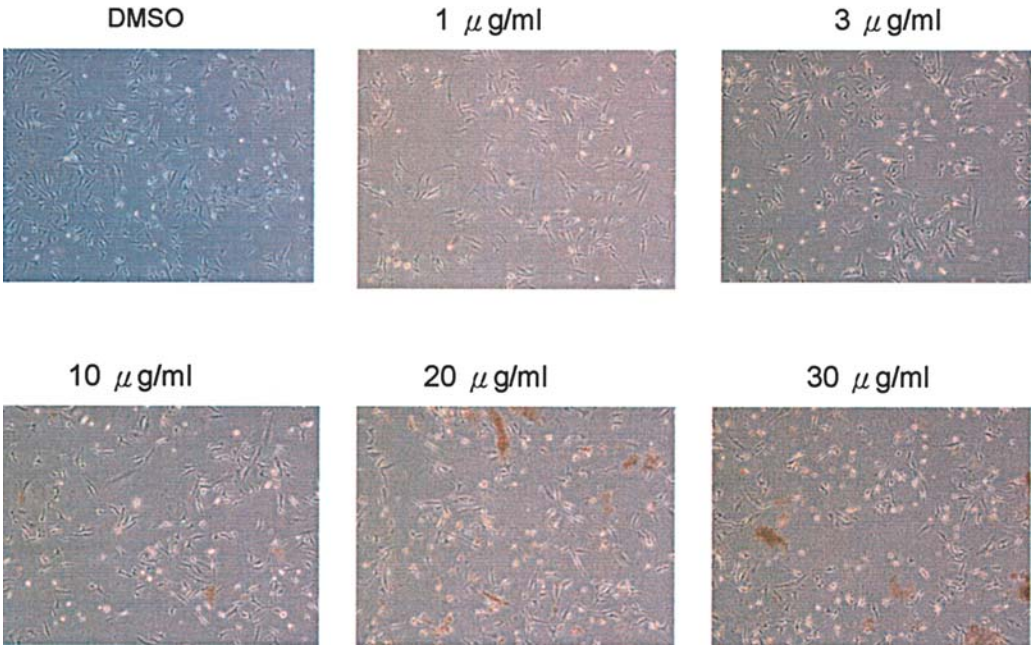

Figure 1. MDA-MB-231 cells were treated with various concentrations of Tan IIA for 24 h. Cells were examined using a contrast-phase microscope (magnification x50) and photographed.

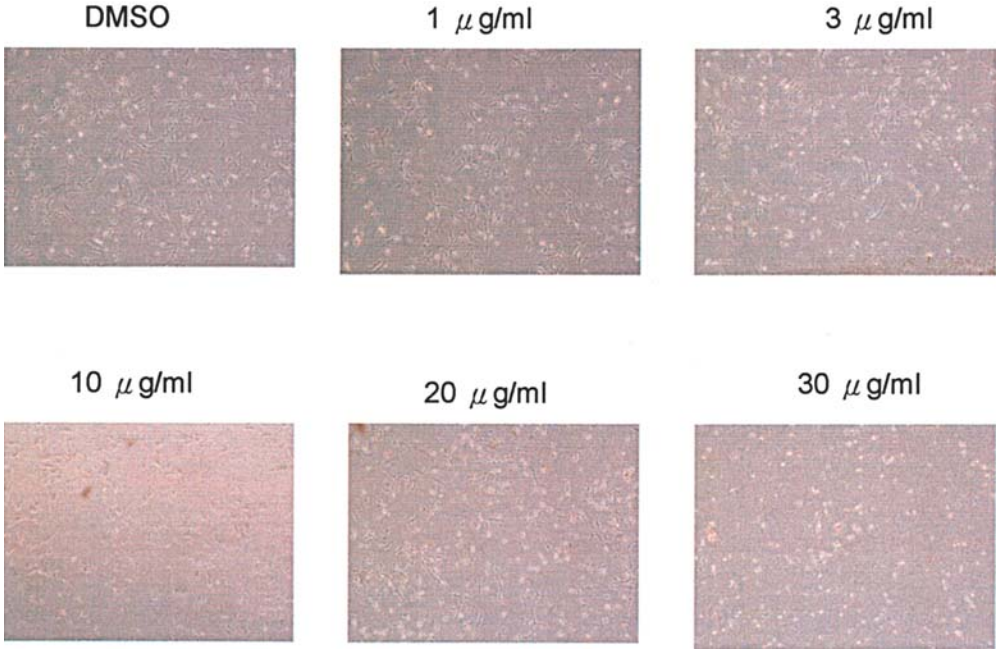

Figure 2. MDA-MB-231 cells were treated with various concentrations of Tan IIA for 48 h. Cells were examined using a contrast-phase microscope (magnification x50) and photographed.

fresh medium per well, containing 10 mM HEPES was then added, 200 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the wells and the plate was incubated for 30 min at 37˚C in dark conditions. The medium was removed and 1000 μl DMSO was added to the wells. Absorbance was measured using an ELISA plate reader at 590 nm.

Cell cycle analysis for MDA-MB-231 cells treated with tanshinone IIA using a flow cytometry assay. The percentage of cells in sub-G1, G0/G1, S and G2/M phases was determined by flow cytometry as described (10). Briefly, 1x10^6 MDA-MB-231 cells/10-cm dishes were incubated with tanshinone IIA at 0, 6 and 12 μg/ml concentrations for 48 h and at 0, 1.25 and 2.5 μg/ml concentrations for 72 h before the cells were harvested by centrifugation. The cells, after being harvested were washed with PBS, then fixed gently (drop by drop) in 70% ethanol (in PBS), in ice, overnight. They were then resuspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100. After 30 min at 37˚C

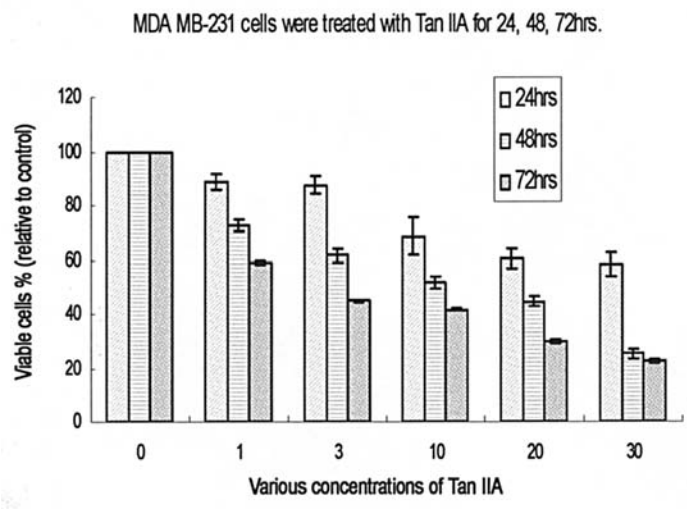

Figure 3. The MDA-MB-231 cells were plated at a density of $1x10^5$ cells/well and grown for 24 h. The various concentrations of tanshinone IIA were added and cells grown for 24, 48, and 72 h, while only DMSO 0.2% (solvent) was added to the control regimen. After 24, 48 and 72 h of culture, the viability was evaluated by MTT assay in triplicate.

in dark conditions, the cells were analyzed using flow cytometry (Becton-Dickinson, San Jose, CA, USA) and equipped with an argon laser at 488 nm. Cell cycle and apoptosis were then determined and analyzed using the ModFit software. The average of the percentage of each phase in the cell cycle was representative of the three independent experiments.

Protein preparation. Approximately $1x10^6$ cells/10-cm dish were incubated with tanshinone IIA at 0, 6 and 12 μg/ml concentrations for 48 h before the cells were harvested by centrifugation. Protein was extracted as described (11). Briefly, cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 0.25% sodium deoxycholate, 1mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4˚C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4˚C and supernatant was collected, aliquoated (50 μg/tube) and stored at -80˚C before being assayed. The protein concentrations were measured using the Bradford method (12).

Western blotting for examining the effect of tanshinone IIA on the expression of Bax, Bcl-xL, p21, caspase-8 and ß-actin in MDA-MB-231 cells. All samples were separated by sodium dodecylsulfate polyacrylamide (SDS-PAGE) gel electrophoresis (10 and 13%) (Bio-Rad Life Science Products, Hercules, CA, USA) as described (11). The SDS-separated proteins were followed by equilibration in transfer buffer [50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS, 20% methanol and electro-transferred to Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA, USA)]. Then the blot was blocked with a solution containing 5% non-fat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl (Sigma Chemical Co.), contained in 0.05% Tween-20 for 1 h. It was then washed and incubated with antibodies to Bax, Bcl-xL, p21, caspase-8 and ß-actin, (Upstate, Lake Placid, NY, USA) at 4˚C overnight. After incubating with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the signal was visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The detection of ß-actin was used as an internal control in all of the data for Western blotting.

Statistical analysis. Values are presented as a mean ± SD. Student's t-test was used to analyze the statistical significance and $p<0.05$ was considered significant for all tests.

Results

Morphological changes of human breast cancer MDA-MB-231 cells in response to various concentrations of tanshinone IIA. The MDA-MB-231 cells were treated with various concentrations (0, 1, 3, 10, 20 and 30 μg/ml) of tanshinone IIA for 24 and 48 h (Figs. 1 and 2). Cells were examined using contrast-phase microscope (x50) and photographed. The results indicated that the number of viable cells decreased as the concentration increased, suggesting that tanshinone IIA induced cell death on MDA-MB-231 cells.

The effects of tanshinone IIA on the viability of MDA-MB-231 cells. The MDA-MB-231 cells ($1x10^5$ cells/well) were plated in L-15 medium + 10% FBS with various concentrations (0, 1, 3, 10, 20 and 30 μg/ml) of tanshinone IIA for 24, 48 and 72h. The cells were collected by centrifugation and the viable cells were determined by MTT assay as previously described. The viable cell percentages relative to control were 88.58±2.85, 87.54±3.10, 68.70±7.06, 60.23±3.50 and 58.23±4.11%, respectively, when cultured with various concentrations of tanshinone IIA (1, 3, 10, 20 and 30 μg/ml) for 24 h. The viable cell percentages relative to control were 72.72±2.50, 61.48±2.47, 51.56±1.94, 44.53±1.69 and 25.15±1.82%, respectively, when cultured with various concentrations of tanshinone IIA (1, 3, 10, 20 and 30 μg/ml) for 48 h. The viable cell percentages relative to control were 59.14±0.75, 44.77±0.29, 41.65±0.34, 29.84±0.66, and 22.61±0.77%, respectively, when cultured with various concentrations of tanshinone IIA (1, 3, 10 and 20 and 30 μg/ml) for 72 h (Fig. 3).The IC_{50} were 34.14±4.55, 11.85±0.29 and 2.40±0.02 μg/ml respectively, when MDA-MB-231cells were treated with tanshinone IIA for different durations (24, 48 and 72 h). The proliferation of colo 205 was obviously inhibited by tanshinone IIA in a dose- and time-dependent manner.

Tanshinone IIA induced apoptosis in MDA-MB-231 cells. The results of FCM showed that the percentages of sub-G1 were 4.32, 9.93 and 11.8%, respectively, when MDA-MB-231 cells were cultured with various concentrations (control, 6 and 12 μg/ml) of tanshinone IIA for 48 h (Fig. 4). The percentages of sub-G1 were 5, 8.92 and 9.26%, respectively, when MDA-MB-231 cells were cultured with various concentrations (control, 1.25 and 2.5 μg/ml) of tanshinone IIA for 72 h (Fig. 5).

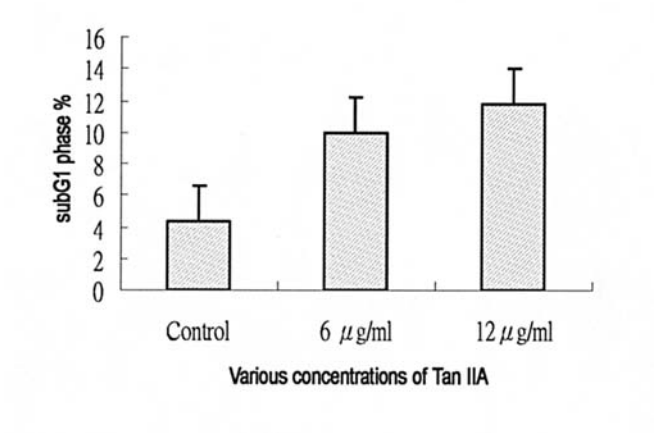

Figure 4. MDA-MB 231 cells were treated with Tan IIA for 48 h. Then the sub-G1 cell cycle percentage was evaluated by FACS.

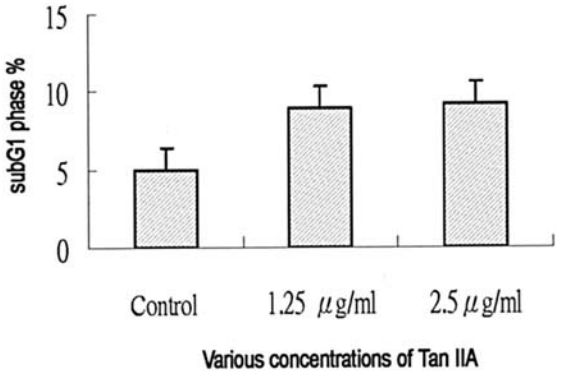

Figure 5. MDA-MB 231 cells were treated with Tan IIA for 72 h. Then the sub-G1 cell cycle percentage was evaluated by FACS.

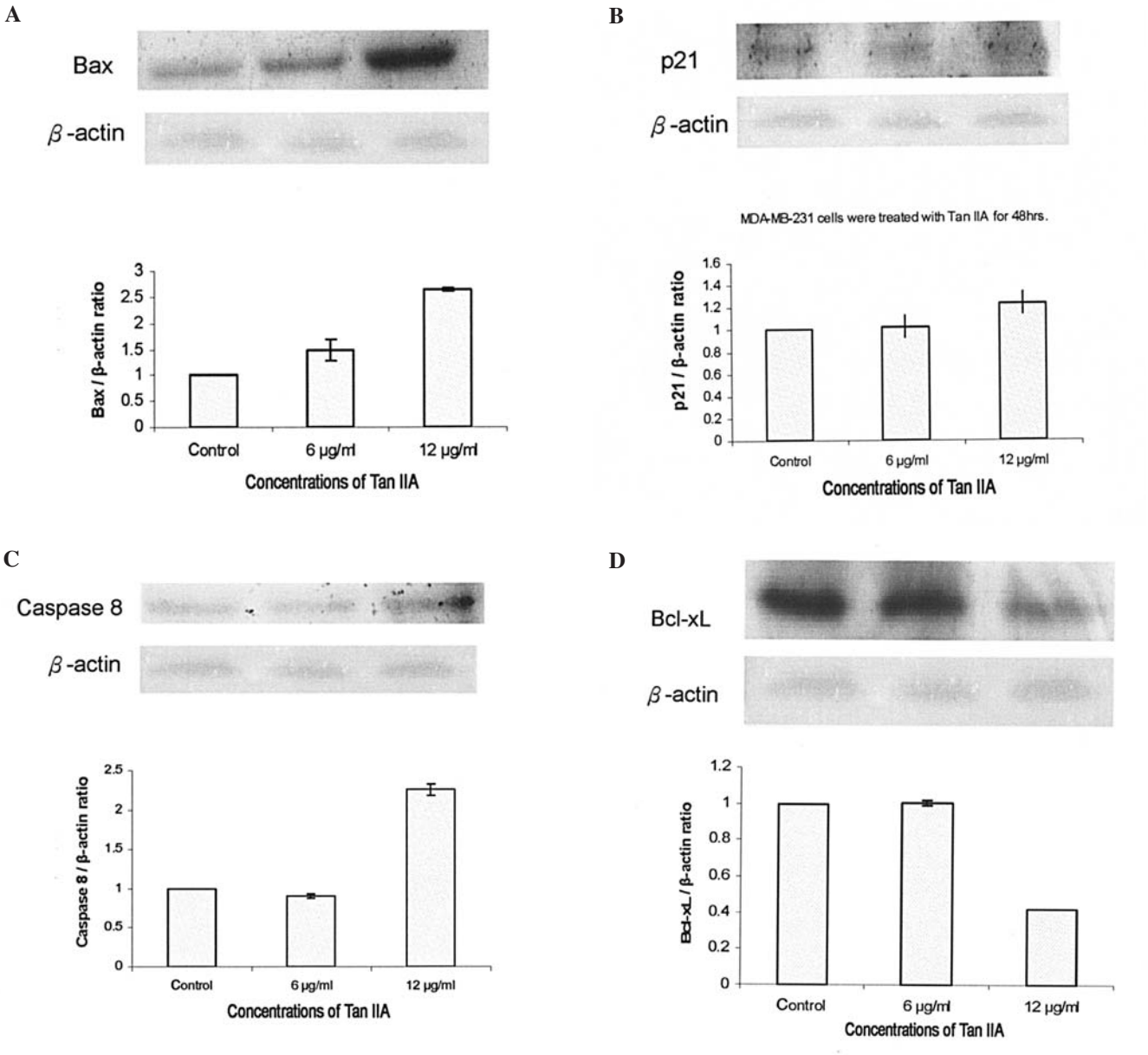

Figure 6. The protein expression of Bax, Bcl-xL, p21, caspase-8 and ß-actin in MDA-MB-231 cells treated with tanshinone IIA (Tan) for 48 h. Tan increased Bax to Bcl-xL ratios and up-regulated the protein expression of p21 and caspase-8 *in vitro*.

Western blot analysis on the effect of tanshinone IIA on Bax, Bcl-xL, p21, caspase-8 and ß-actin in MDA-MB-231 cells. MDA-MB-231 cells were harvested in the presence of tanshinone IIA for Western blotting and the results indicated that tanshinone IIA increased the expression of Bax, p21 and caspase-8 (Fig. 6c). Tanshinone IIA decreased the protein expression of Bcl-xL (Fig. 6d).

Discussion

The present study showed that the proliferation of MDA-MB-231 cells was inhibited by tanshinone IIA in a dose- and time-dependent manner. This finding is in agreement with others, demonstrating that tanshinone IIA induces cytotoxicity in MDA-MB-231 cells and induces apoptosis in human breast cancer MDA-MB-231 cells (8). It is well-documented that an increased Bax and decreased Bcl-xL protein expression will induce cancer cell apoptosis (13-15). Although many experiments have demonstrated that tanshinone IIA induces apoptosis in other cancer cell lines (8,16), this is the first study showing that tanshinone IIA inhibits the proliferation of human breast cancer cells through inducing apoptosis by increasing Bax to Bcl-xL ratios *in vitro*. Our results also show that tanshinone IIA increases the protein levels of p21 and caspase-8 in human breast cancer MDA-MB-231 cells. It is well documented that p21 (WAF1 and Cip-1) has the potential to induce G1 arrest (17) and apoptosis (18,19). One of the mechanisms by which tanshinone IIA induces apoptosis in human breast cancer MDA-MB-231 cells may be through the up-regulation of expression of p21, inducing apoptosis. Another mechanism may be through the membrane receptor pathway. Taken together, these data suggest that tanshinone IIA has significant therapeutic potential for human breast cancer.

Acknowledgements

This study was supported by the grant TCRD96-10 from the Research Section of the Buddhist Tzu Chi General Hospital, Hualien, Taiwan, R.O.C. This study was partially supported by The Chen-Han Foundation for Education.

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